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THE SURVIVAL OF VIRUSES AT LOW TEMPERATURES

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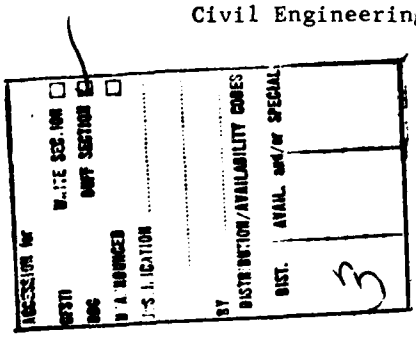
ABSTRACT

Sanitary facilities in polar regions are often very primitive, and disposal practices may be haphazard and random. The resultant potential hazard to the health of personnel in the polar environment has received little attention and is generally underestimated. Consequently, a laboratory investigation was instigated on the survival of viruses at low temperatures. The results of this preliminary study show that viruses in sewage frozen at temperatures as low as -40°C remain viable and may even increase in number. After 4 months of storage at temperatures as low as -33°C, 10 to 20% of the viruses remained infective. That any viruses remained for any period of time is significant since very minute amounts of enteroviruses can initiate disease in humans. Because so little is known on the survival of viruses at low temperatures, additional research is needed to delineate more completely the circumstances under which viruses do survive and, hence, constitute a menace to health.



INTRODUCTION

The extreme environment and remote location typical of most polar camps create significant problems with respect to the provision of sanitary facilities. Despite the recent increase in population in polar regions, sanitary facilities are often very primitive, and sewage disposal practices may be haphazard and random.

The potential hazard to the health of personnel in the polar environment resulting from lack of treatment and random disposal of raw sewage has received little attention, and is generally underestimated. The Naval Civil Engineering Laboratory (NCEL) is currently trying to improve polar sanitary conditions and practices; current efforts are concentrating on support of the United States operation in Antarctica, although most of the results are applicable to the Arctic also. Based on the literature search of LeGros and Drobny (1966), an investigation of the survival of viruses at low temperatures was initiated; this technical note summarizes the initial results.

BACKGROUND

LeGros and Drobny (1966) cite considerable literature on the potential danger of spreading enteric viral infections by sewage disposal methods prevalent in antarctic camps. "Honey buckets" and conventional toilets connected to insulated pipes are emptied into open dumps, where their contents are quickly frozen. The dumps, which generally are not marked, subsequently are covered with snow, and often their location becomes unknown. Ice and snow from such dumps may ultimately, though inadvertently, be melted to provide a camp's water supply. The human wastes contaminating such water may contain as many as 70 different viruses pathogenic to man, and thus act as an agent of disease transmission. Since temperatures during the melting process rarely exceed $10^{\circ}C$, the well-known inactivation of viruses by high temperatures is negated. Of note here is the report of Havens (1945) that the exposure of infectious hepatitis virus, an intestinal pathogen transmitted in sewage, to $56^{\circ}C$ for 30 minutes failed to render the virus inactive.

Among the better-known waterborne enteroviruses, in addition to that causing infectious hepatitis, are polio virus, coxsackie virus, and meningitis virus; adenovirus, a respiratory virus, is also transmitted by water.

Infectious Hepatitis Virus

The enterovirus most commonly responsible for outbreaks in human populations is that of infectious hepatitis. Incidence of this illness has increased dramatically in recent years, and outbreaks annually occur throughout the world. In 1955-56, a water supply contaminated by sewage was responsible for 30,000 to 50,000 cases in New Delhi, India. In California, with a high standard of living, over 5,000 cases of infectious hepatitis occurred in the first 9 months of 1967 (Anon, 1967). This does not imply that all were waterborne, but the figures do illustrate the endemic nature of the virus and the potential for catastrophe. Of importance also is the fact that the incidence can be high in troops and is not uncommon in overcrowded dwellings.

Characteristically, enteroviruses may survive outside the body for varying periods of time, and survival is enhanced by lowered temperatures. Neefe and Stokes (1945) have demonstrated that the infectious hepatitis virus can survive storage in well water for 4 to 10 weeks. Weber (1959) reports the survival of the virus for a month in ground waters. Of importance is the effectiveness of this virus in high dilution; Stinger (1955) determined that human volunteers contracted the disease after ingestion of 10^{-12} dilution of infected feces. A less direct mode of transmission than via contaminated water is that of human infection following consumption of shellfish from polluted waters (Jensen, 1961).

Infectious hepatitis is more resistant to free chlorine in the water (Clark and Chang, 1959) than are coliform or enteric pathogenic bacteria, elimination of which signifies sufficient chlorination for sanitization. Water is routinely checked for the presence of coliform bacteria, whose presence implies human pollution, and is not tested for the presence of viruses.

The relatively high resistance of hepatitis virus to heat has been previously cited. The authors, however, found no reference dealing with the effect of low temperatures on infectious hepatitis virus other than generalizations that low temperatures extended the period of infectivity.

Coxsackie Virus

The coxsackie virus is small and spherical, measuring 25 to 30μ in diameter. It causes a number of diseases ranging from slight fever to an illness that is clinically indistinguishable from poliomyelitis. Coxsackie viruses, like infectious hepatitis viruses, can survive storage outside the host at 8 to 10^{o}C for 30 weeks (Gilcreas and Kelley, 1955). When sewage and raw river water containing the virus were stored at the same temperature, survival in sewage was for a period of 17 weeks and in river water, 3-1/2 weeks (Clarke, et al, 1952).

Poliomyelitis Virus

Poliomyelitis is also a small spherical virus (25 to 30μ in diameter), producing symptoms ranging from the subclinical to the extreme of paralysis and aseptic meningitis. A report in the British Medical Journal (Anon, 1957) states that a polio patient may excrete, in a single gram of feces, sufficient viruses to infect 1,000,000 monkeys. The excretion of viruses may continue for periods up to 12 weeks.

Polio virus can remain viable outside the host in water or refrigerated feces for several weeks (Mohlman, 1943; Krumbiegel, 1944). Soviet workers report that, at room temperature, polio virus remains infective in human feces for over 24 weeks. In milk, survival persisted for 4-1/2 weeks at room temperature, and for 21-1/2 weeks at 10°C. In experimentally polluted water, polio virus remained viable for at least 26 weeks (Levkovich, 1957). Rhodes (1950) noted the survival of polio virus for 17 weeks in water at a temperature of 4°C.

Like the virus of infectious hepatitis, the polio virus can be transmitted by shellfish. Passage of the virus through the mussel does not alter its pathogenicity. Crovari (1958) found that if mussels were left for 3 hours in polluted seawater, they became contaminated; he also isolated polio viruses from contaminated mussels after washing them with running seawater for 24 hours.

Low Temperature Preservation

A review of the literature as cited in this report and that of LeGros and Drobny (1966) reveals a paucity of experimental evidence concerning the effects of low temperatures on viruses. Freeze-drying, the preservation of viruses and their products, such as vaccines and sera for medical uses, however, is one area of research which has considered the survival of viruses at low temperatures. This technique involves the freezing of hydrated viruses so as to survive the conversion of viral water to ice, the removal of the ice by sublimation, and ultimately, the reactivation of the viruses by return of the water to the liquid condition. In is profitable to examine the techniques that have evolved, noting the applicability of results to the problem of virus survival in sewage of polar regions.

The initial objective of the virologist is to grow the viruses under suitable conditions so that large titers* will result. This task is frequently difficult, or at times impossible, because viruses are obligate, intracellular parasites that are host specific. Despite its ubiquitous nature, the infectious hepatitis virus has not been grown in the laboratory. The second task is to concentrate and purify the viruses by selective removal of non-viral materials, such as the

*Titer - The number of viral particles per unit volume.

host tissue, and any nutritive materials used. Techniques such as chemical precipitation, ultra-centrifugation, and ion exchange are successful.

Once grown and isolated, the viruses may be preserved by freezing, a procedure which is apparently affected by many variables. For example, the temperature of freezing may be influential. Greiff, et al (1954) demonstrated that polio virus, measles virus, and influenza virus showed significant decrease in titer when frozen at -20 and -40°C, though no such decrease was noted at -60 and -76°C. The rate of freezing may also be of significance. Greiff and his associates noted that when polio virus was slowly frozen, the survival rate was significantly lower than when rapidly frozen at the same temperature. With measles and influenza viruses, however, different rates of freezing produced no differences in titer. The rate of freezing at a given temperature is primarily a function of the volume to be frozen. The present authors found no specific references relative to the importance of the thawing temperature on the survival of viruses. Meryman (1966) states that with bacteria and other biological cells, however, the temperature of thawing after slow freezing is irrelevant. But, following rapid freezing, rapid thawing appears to be necessary for survival.

Changes in virus titers after cycles of freezing and thawing have been investigated. Sanderson (1925) noted no change in titers of bacterial viruses after 10, 15, or 20 consecutive cycles of freezing and thawing. Greiff, et al (1954) observed that when polio viruses, which are enteroviruses, were exposed to 16 cycles of freezing at -76°C and thawing at 10°C, no significant decrease in titer occurred.

The length of time and the temperature of storage are pertinent to the present study. Greiff and Rightsel (1966) state that there is no loss in titer of polio virus slowly frozen at temperatures of -20, -40, and -76°C and stored for 26 weeks at -20, -40, and -65°C respectively. The opposite was true with measles and influenza viruses. Turner and Fleming (1939), and Horsfall (1940) demonstrated that viruses in fluids of unknown composition, when frozen and stored at identical temperatures, showed no appreciable loss of titer after several (6 to 12) months. Rabies virus stored at -4°C maintained virulence for 104-1/2 weeks, and preparations stored at -20°C were virulent after 126 weeks (Lepine and Sautter, 1941). Meryman (1963) has concluded, however, that in all living cells and tissues, physical and chemical activities are reduced to negligible levels at temperatures below -130°C.

Of particular significance to the present study is the role of additives as protective agents in preventing the deleterious effects of freezing on viruses. Protective compounds in use are:

1. Protein adjuvants (gelatin, serum, peptone, etc.)
2. Skim milk
3. Sugars (lactose, sucrose, glucose, etc.)
4. Glycerol
5. Allantoic fluid
6. Dimethyl sulfoxide

Greiff and Rightsel (1966) report that dimethyl sulfoxide protects influenza and measles viruses, and speculate that the effect results from the shortened freezing time.

All of the above-cited evidence pertains exclusively to the goal of preserving viruses utilizing low temperatures. The results of this experimentation have a direct and important relationship to the survival of enteroviruses in sewage at polar regions. We may assume that any individuals infected with enteroviruses, having clinical or subclinical symptoms or even asymptomatic carriers, may excrete significant amounts of the pathogens. The viruses may be protected from deactivation by the presence of body fluids and excreta which are analagous to the protective additives used experimentally. Similarly, the optimal temperatures of freezing and thawing successfully employed for long-term preservation in the laboratory are encountered in polar regions. It is valid to conclude that enteroviruses may be found in sewage, they may be preserved for periods of time in ice, and we may speculate that when the ice is melted and used for domestic purposes, the viruses may be transmitted to susceptible individuals. Speculation, however, provides no experimental definitive evidence concerning the survival of viruses in human discharges that have been frozen and thawed. This report presents the results of preliminary experiments of the effects of low temperatures on viruses in sewage.

MATERIALS AND METHODS

The present study utilizes bacterial viruses having the bacterium, Escherichia coli, strain B, as the host organism. This bacterium and the virus are omnipresent in human sewage and waters contaminated by the sewage. Indeed, E. coli is the organism whose presence in domestic water supplies is officially used as an indicator of pollution. The bacterial host is readily isolated, grown, and maintained in the laboratory, and the cost is minimal. Neither the host bacterium nor the virus is pathogenic.

Bacterial viruses were utilized in lieu of enteroviruses because use of the latter presumes the comparatively complex and expensive maintenance of tissue cultures or susceptible laboratory animals. In addition, they are human pathogens. Enteroviruses are not omnipresent in feces or sewage, and when present, their detection and isolation is comparatively difficult. Earlier in this report, reference was made to the existing inability to grow infectious hepatitis virus in the laboratory.

Of paramount importance in this study is the awareness that E. coli virus and enteroviruses have a common structure and chemical composition, a central core of nucleic acid surrounded by a coat, or capsid, of protein. It is reasonable to conclude that information derived from studying viruses of bacteria associated with sewage is applicable to enteroviruses in sewage; subsequent research, however, should utilize the latter viruses.

Sewage was collected from the influent line of the city of Ventura biofiltration plant. The titers of controls were determined at the time of collection. Aliquots of sewage (10 to 500 ml) were placed in cylindrical polypropylene containers having a diameter of 8.9 cm. The samples, fitted with thermocouples, were placed in domestic freezers at -18, -33, and -40°C. One 10-ml aliquot of sewage in a stainless steel beaker with a diameter of 5.4 cm was placed in liquid nitrogen at -126°C. After freezing, the samples were thawed at approximately +20°C, and virus titers were determined.

Titers were determined by plaque assay (Adams, 1959). Liquid sewage was spun in a refrigerated centrifuge, International model PR-1 for 10 minutes at 25,000 G with a temperature of -8°C. The liquid was filtered through a membrane filter with a pore size of 0.45μ. An aliquot, 0.1 ml to 1.0 ml, of the filtrate was added to 2.0 ml of "soft" agar that had been inoculated with 0.1 ml of a 9-hour broth culture of Escherichia coli, strain B. The "soft" agar consisted of 0.5% tryptone, 0.5% NaCl, and 0.6% agar. The "soft" agar containing bacteria and viruses was poured over "hard" agar and incubated at 37°C for 24 hours. The "hard" agar was composed of nutrient agar with 0.5% NaCl. "Hardening" consisted of pouring the heated liquid agar into petri dishes which were placed in a warm environment for 24 hours prior to use. After incubation of the plates of "hard" agar overlain with inoculated "soft" agar, the plaques formed were counted and the titer determined as viral particles per ml of sewage.

A plaque represents a single virus particle and is counted as such. The entire surface of the agar is covered with a whitish bacterial growth, and plaques are circular clear areas devoid of bacteria (Figure 1). The bacterial virus used in this study is "tadpole" shaped (Figure 2), with a polyhedral "head" and cylindrical tail. The inner portion, or core, consists of the nucleic acid, deoxyribonucleic acid (DNA), and the outer portion, or capsid, consists of proteins. The capsid terminates in six fibrils which are associated with attachment of the virus to a susceptible bacterial cell (Figure 3). At the point of attachment, the bacterial cell wall is dissolved, and viral DNA enters the bacterial cell, and the capsid remains outside as an empty inert shell. The DNA of the virus disappears, as such, inside the bacterial cell. Accordingly, the virus is said to be in eclipse, a period of approximately 12 minutes duration. During this time, viral DNA takes over the synthetic mechanisms of the bacterial cell, causing them to synthesize 20 to 200 units of viral DNA. This synthesis is followed by formation of a capsid

enveloping each strand of viral DNA, and within 30 minutes after attachment of the virus to the outside of the bacterial cell, the cell wall ruptures and the newly formed viruses are set free to begin the cycle anew. The bacterium is said to have undergone lysis, and repeated cycles are macroscopically manifest as plaques. The bacterial viruses lysing E. coli are typed T_1, T_2, T_3, etc., according to size, and plaque sizes are inversely proportional to virus sizes.

RESULTS

The time required to freeze three of the aliquots of sewage is illustrated in Figures 4 through 6. The curves are similar to those discussed by Greiff and Rightsel (1966) and characteristically, have three pertinent portions - supercooling, the freezing plateau, and lastly, the resumption of cooling and the completion of freezing. The significant difference in all curves is the length of time the samples were at the freezing plateau.

The titers of viruses from samples before and after freezing are given in Table 1. From this, it can be seen that when a small sample was frozen at -18, -23, -32, -33, and -40°C and immediately thawed, the titer was relatively unchanged; indeed, it increased. In like manner, when a larger sample was frozen at -32°C, thus lengthening the freezing and thawing periods, the titers were not significantly lessened and, in the case of sample 4, increased.

A 4-month storage period in ice at temperatures of -18, -23, and -33°C seemed to inactive 80 to 90% of the viruses; that any viruses can remain infective for any period of time in ice is, however, of significance, especially considering the high dilutions and low titers of enteroviruses that initiate disease in humans. Other samples are presently in freezers and will be examined after longer storage periods.

Freezing viruses in liquid nitrogen appears to be detrimental to the viruses and without great bearing on the present study, for the temperatures of the polar regions do not approach -126°C.

SUMMARY

A potential health hazard and source of pollution exists in polar regions where human waste is discarded in unmarked dumps to be covered with snow, and the location lost. As many as 70 different viruses pathogenic to man may survive in this waste for years at temperatures ranging from +56 to -76°C. A paucity of experimental evidence concerning the effects of low temperatures on viruses exists; no comprehensive investigation of the survival of viruses, and especially enteroviruses, has been conducted at low temperatures. The results of this preliminary investigation indicate that viruses do survive

Table 1. Titers of Viruses From Samples Before and After Freezing

Sample No.	Temp. of Freezing (°C)	Quantity Frozen (ml)	Freezing Time (min)	Titer[a] Control A (Viruses/ml)	Titer[b] Control B (Viruses/ml)	Titer[c] Exp. A (Viruses/ml)	Titer[d] Exp. B (Viruses/ml)
1	-18	25	20	1,189	---	2,665	570[e]
2	-23	25	20	1,189	---	3,740	378[f]
3	-32	500	180	585	249	155	---
4	-32	500	180	3	8	453	---
5	-32	500	180	2,598	2,454	2,459	---
6	-32	500	180	4,417	3,883	3,897	---
7	-32	20	15	1,125	---	1,311	---
8	-33	25	20	1,189	---	1,860	319[g]
9	-40	25	5	1,189	---	1,190	---
10	-126	10	1	1,970	---	495	---

a - Titer of control at time when freezing of sample is commenced
b - Titer of control at time when frozen sample has thawed.
c - Titer of samples after freezing and thawing.
d - Titer of samples after freezing and stored for specific cited periods.
e - Stored 4 months at -18°C.
f - Stored 4 months at -23°C.
g - Stored 4 months at -33°C.

at temperatures as low as -40°C, and even increase in number under some circumstances. Additional research is needed to delineate more completely the circumstances under which viruses do survive and hence constitute a menace to health. Such research should include:

1. A large number of samples, with emphasis on:
   a. A large number of different freezing temperatures.
   b. A large number of different thawing temperatures.
   c. Freezing and storing at different temperatures for varying lengths of time.
   d. Different volumes of sewage.
   e. Effects of light and darkness during freezing and/or thawing.
2. Samples of sewage of known history from polar regions.
3. The use, ultimately, of enteroviruses rather than bacterial viruses.

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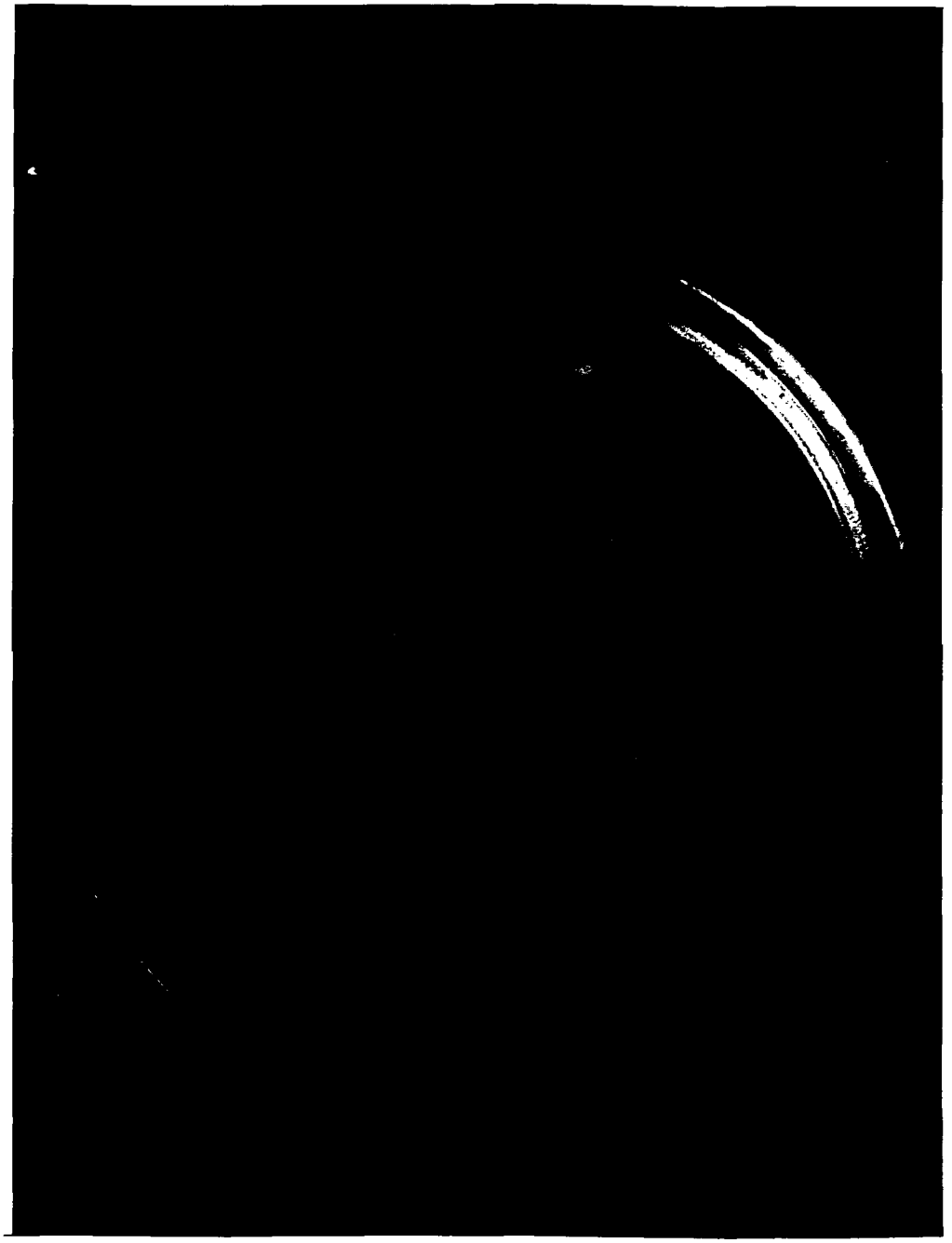

Figure 1. Circular plaques formed by lysis of bacteria by viruses.

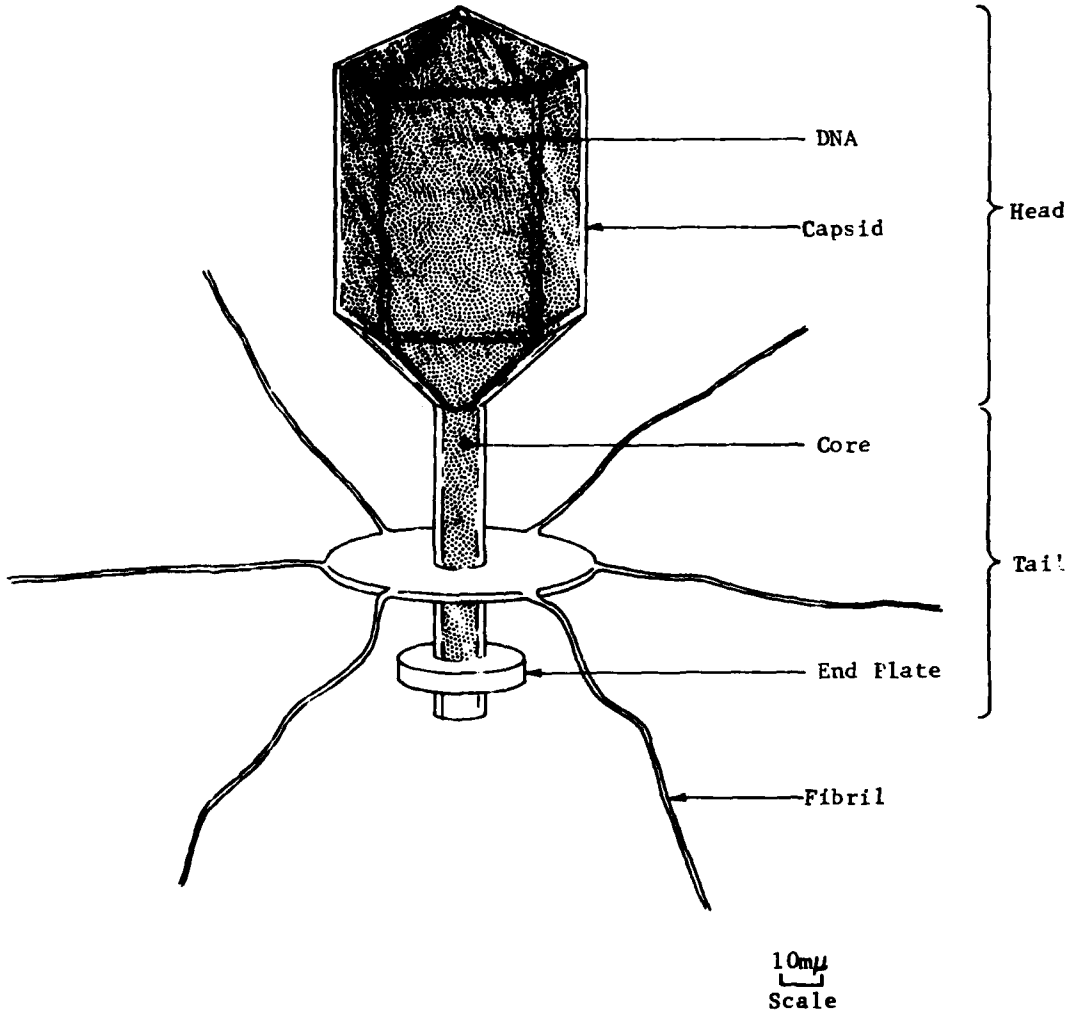

Figure 2. Diagram of structure of Escherichia coli bacteriophage (T_2).

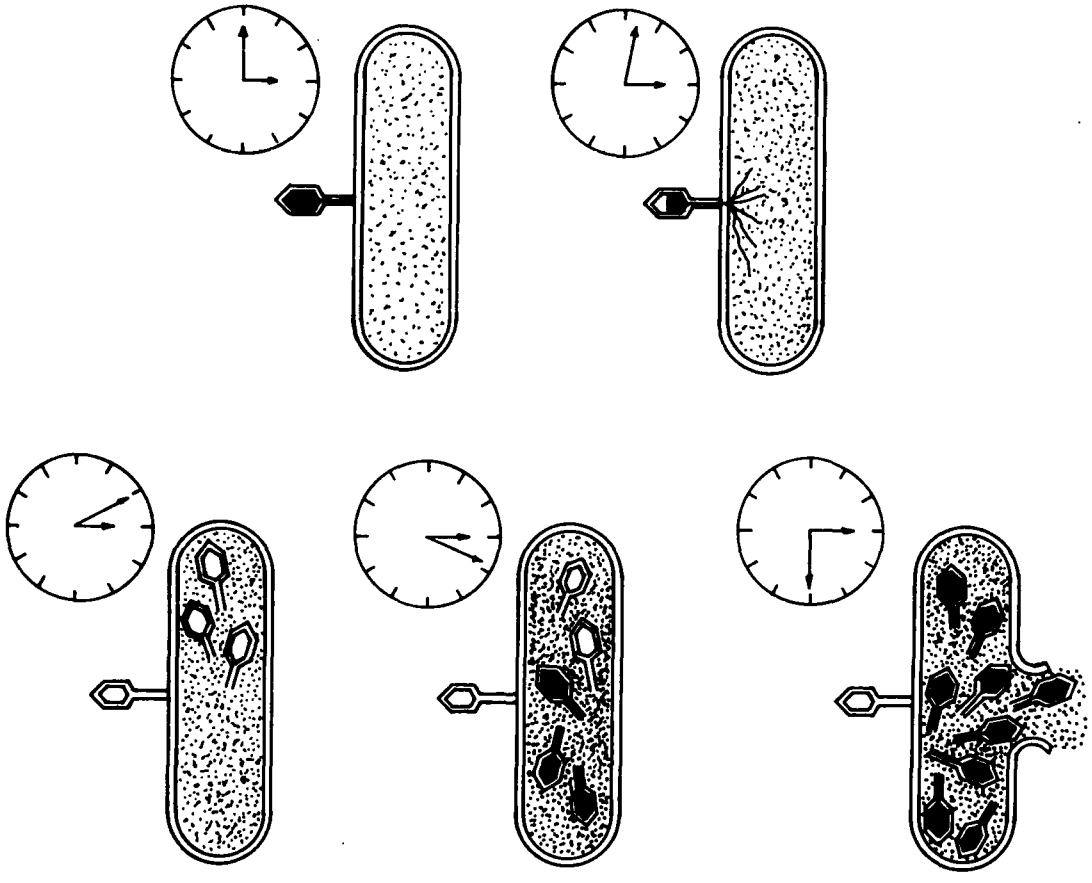

Figure 3. Successive stages in the reproduction of a bacterial virus (after Stent, 1953).

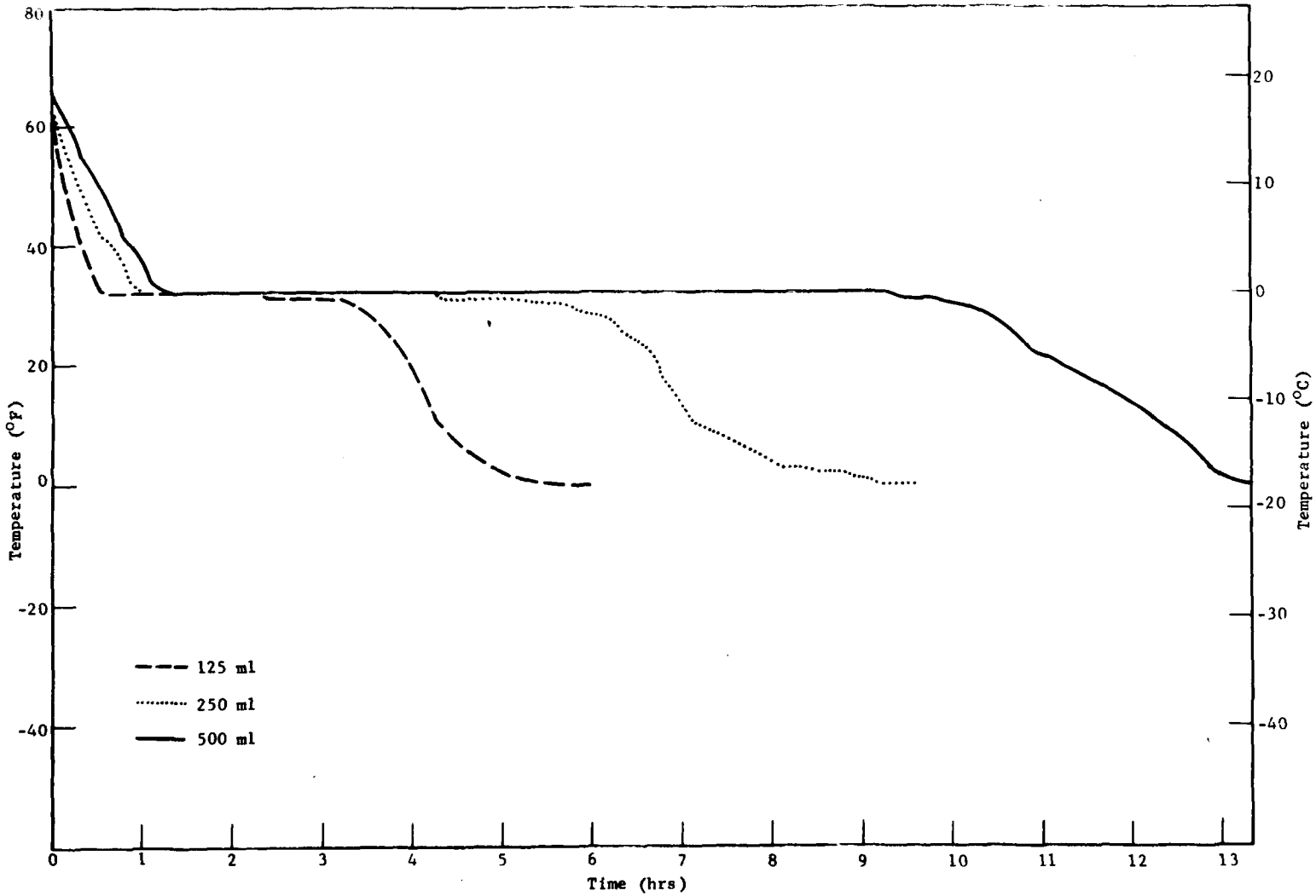

Figure 4. Time required to freeze three aliquots of sewage at -18°C.

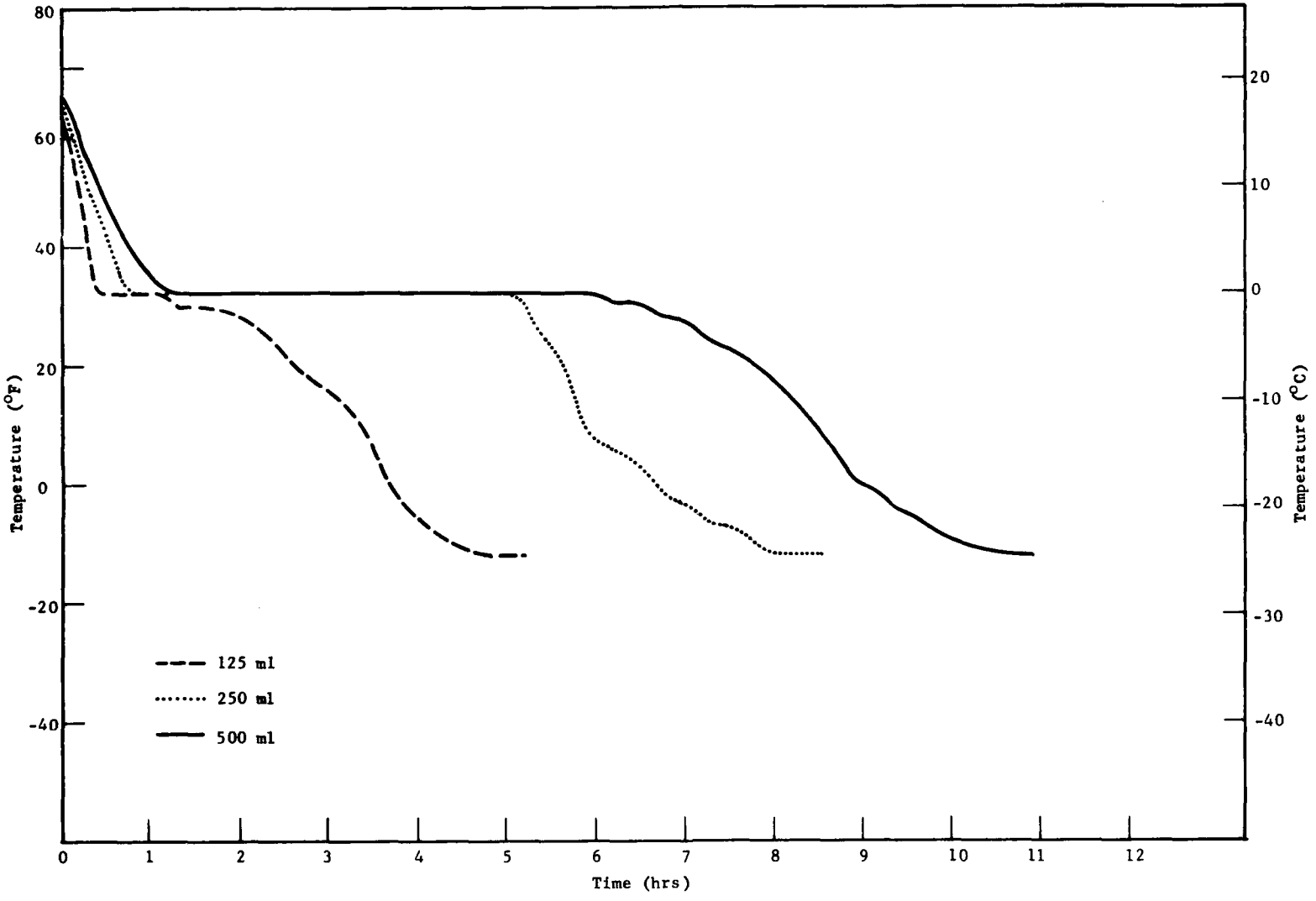

Figure 5. Time required to freeze three aliquots of sewage at -24°C.

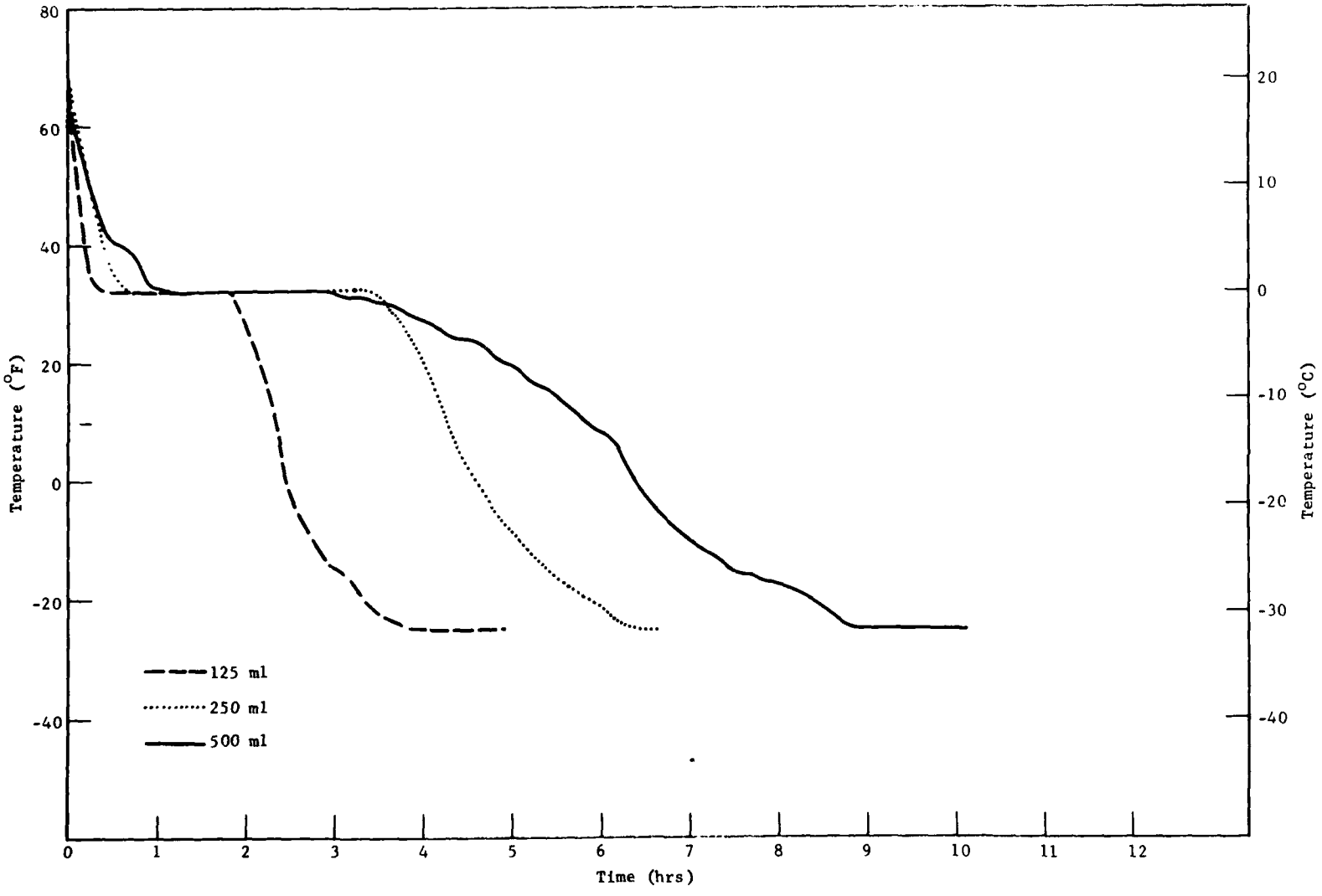

Figure 6. Time required to freeze three aliquots of sewage at -32°C.

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13 ABSTRACT

Sanitary facilities in polar regions are often very primitive, and disposal practices may be haphazard and random. The resultant potential hazard to the health of personnel in the polar environment has received little attention and is generally underestimated. Consequently, a laboratory investigation was instigated on the survival of viruses at low temperatures. The results of this preliminary study show that viruses in sewage frozen at temperatures as low as -40°C remain viable, and may even increase in number. After 4 months of storage at temperatures as low as -33°C, 10 to 20% of the viruses remained infective. That any viruses remained for any period of time is significant since very minute amounts of enteroviruses can initiate disease in humans. Because so little is known on the survival of viruses at low temperatures, additional research is needed to delineate more completely the circumstances under which viruses do survive and, hence, constitute a menace to health.

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